DOCKET NO.: CHIR-0315 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Covacci *et al.***

Serial No.: **not yet assigned** Group Art Unit: **not yet assigned**

Filed: **Herewith** Examiner: **not yet assigned**

For: **HELICOBACTER PYLORI CYTOTOXIN PROTEINS USEFUL FOR VACCINES AND DIAGNOSTICS**

EL568088980US

EXPRESS MAIL LABEL NO:
DATE OF DEPOSIT: August 2, 2001

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

PRELIMINARY AMENDMENT

Applicants respectfully request that the application be amended as follows.

IN THE SPECIFICATION:

At page 1, after the title of the application, please insert the following subtitle and paragraph.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Application Serial No. 09/360,934, filed July 26, 1999, which is a divisional of U.S. Application Serial No. 08/471,491, filed June 6, 1995, now issued as U.S. Patent No. 6,090,611, which is a divisional of U.S. Application Serial No. 08/256,848, filed October 21, 1994, now abandoned, which is a U.S. national phase application of PCT/EP93/00472, filed March 2, 1993 which also claims priority to PCT/EP93/00158, filed January 25, 1993, both of which PCT applications claimed priority benefit of Italian Application Serial No. FI 92 A 000052, filed March 2, 1992. PCT/EP93/00472 was published in English and

PCT/EP93/00158 was abandoned prior to publication. The entire contents of each application is incorporated herein by reference.

At page 4, please replace the entire **Brief Description of the Drawings** section with the following replacement section.

Figs. 1A, 1B and 1C (SEQ ID NO:2) comprise the nucleotide sequence for the cytotoxin (CT) protein.

Fig. 2 (SEQ ID NO:3) is the amino acid sequence for the cytotoxin (CT) protein.

Fig. 3 is a map of the *cai* gene for the CAI protein and summary of the clones used to identify and sequence this gene.

Figs. 4A through 4F (SEQ ID NO:4 and SEQ ID NO:5) comprise the nucleotide and amino acid sequences of the CAI antigen. The numbers along the left-hand margins of Figs. 4A, 4C, and 4E designate the amino acid positions, and the numbers along the right-hand margins of Figs. 4B, 4D, and 4F designate the nucleotide positions.

Figs. 5A, 5B, and 5C (SEQ ID NO:7 and SEQ ID NO:6) comprise the nucleotide and amino acid sequences of the heat shock protein (hsp).

Replace the paragraph spanning from page 3, line 31 to page 4, line 6 with the following replacement paragraph.

The present invention describes nucleotide and amino acid sequences for three major *H. pylori* proteins. Specifically, these are the cytotoxin, the "Cytotoxin Associated Immunodominant" (CAI) antigen, and the heat shock protein. None of the complete amino acid sequences for these proteins has been known, nor have their genes been identified. The present invention pertains to not only these purified proteins and their genes, but also recombinant materials

associated therewith, such as vectors and host cells. The present invention provides cytotoxin polypeptides that exhibit substantially no toxicity, or substantially reduced toxicity. The present invention also provides CAI and heat shock polypeptides that exhibit no functional contribution to toxicity, or a substantially reduced functional contribution to toxicity. The understanding at the molecular level of the nature and the role of these proteins and the availability of recombinant production has important implications for the development of new diagnostic for *H. pylori* and for the design of vaccines that may prevent *H. pylori* infection and treat disease.

Replace the paragraph spanning from page 49, line 30 to page 50, line 9 with the following replacement paragraph.

DNA manipulation was performed using standard procedures. DNA sequencing was performed using Sequenase 2.0 (USB) and the DNA fragments shown in Fig. 3 subcloned in Bluescript KS+. Each strand was sequenced at least three times. The region between nucleotides 1533 and 2289, for which a DNA clone was not available, was amplified by PCR and sequenced using asymmetric PCR, and direct sequencing of amplified products. The overlapping of this region, was confirmed by one and double side anchored PCR: an external universal anchor (5'-GCAAGCTTATCGATGTCGACTCGAGCT-3'(SEQ ID NO:1)/5'-GACTCGAGTCGACATCGA-3' (SEQ ID NO:8)) containing a protruding 5' HindIII sequence, and the recognition sites of ClaI, SalI, XhoI, was ligated to primer-extended DNA and amplified. A second round of PCR using nested primers was then used to obtain fragments of DNA suitable for cloning and sequencing. DNA sequence data were assembled and analyzed with the GCG package (Genetics Computer Group, Inc., Madison, WI) running on a VAX 3900 under VMS. The GenBank and EMBL databases were examined using the EMBL VAXcluster.

Replace the paragraph spanning from page 52, line 15 to page 53, line 9 with the following replacement paragraph.

The *cai* gene coded for a putative protein of 1147 amino acids, with predicted molecular weight of 128012.73 Daltons and an isoelectric point of 9.72. The basic properties of the purified protein were confirmed by two dimensional gel electrophoresis. The codon usage and the GC content (37%) of the gene were similar to that described for other *H. pylori* genes (13, 26). A putative ribosome binding site: AGGAG, was identified 5 base pairs upstream from the proposed ATG starting codon. Computer search for promoter sequences of the region upstream from the ATG start codon, identified sequences resembling either -10 or -35 regions, however, a region with good consensus to an *E. coli* promoter, or resembling published *H. pylori* promoter sequences was not found. Primer extension analysis of purified *H. pylori* RNA showed that 104 and 214 base pairs upstream from the ATG start codon there are two transcriptional starts sites. Canonical promoters could not be identified upstream from either transcriptional *E. coli* is also recognizing a promoter in this region, however, it is not clear whether *E. coli* recognizes the same promoters of *H. pylori* or whether the *H. pylori* DNA that is rich in A-T provides *E. coli* with regions that may act as promoters. A rho independent terminator was identified downstream from the stop codon. In Fig. 4, the AGGAG ribosome binding site and terminator are underlined, and the repeated sequence and motif containing 6 asparagines are boxed. The CAI antigen was very hydrophilic, and did not show obvious leader peptide or transmembrane sequences. The most hydrophilic region was from amino acids 600 to 900, where also a number of unusual features can be observed: the repetition of the sequences EFKNGKNKDFSK (SEQ ID NO:9) and EPYIA (SEQ ID NO:10), and the presence of a stretch of six contiguous asparagines (boxed in Fig. 4).

Replace the paragraph at page 60, spanning lines 15 to 29 with the following replacement paragraph.

The purified fusion protein was tested by Western blot using sera of patients infected by *H. pylori* and affected by atrophic and superficial gastritis, and patients with duodenal and gastric ulcers: most of the sera recognized the recombinant protein. However, the degree of recognition greatly varied between different individuals and the antibody levels did not show any obvious correlation with the type of disease. In addition, antibodies against *H. pylori* antigens and in particular against hsp protein were found in most of the 12 sera of patients affected by gastric carcinoma that were tested. Although *H. pylori* hsp recognition could not be put in relation with a particular clinical state of the disease given the high conservation between *H. pylori* hsp and its human homolog, it is possible that this protein may induce autoimmune antibodies cross-reacting with the human counterpart. This class of homologous proteins has been implicated in the induction of autoimmune disorders in different systems. The presence of high titers of anti-*H. pylori* hsp antibodies, potentially cross-reacting with the human homolog in dispeptic patients, suggests that this protein has a role in gastroduodenal disease. This autoreactivity could play a role in the tissue damage that occurs in *H. pylori*-induced gastritis, thus increasing the pathogenic mechanisms involved in the infection of this bacterium.

Please insert the following Abstract after page 66.

ABSTRACT

This invention provides polypeptides of *Helicobacter pylori* cytotoxin protein. The invention also provides prophylactic and therapeutic vaccines comprising the polypeptides of *Helicobacter pylori* cytotoxin protein, and methods for their preparation.

IN THE CLAIMS:

Please cancel claims 1 - 37 without prejudice, and add new claims 38 - 44 as follows.

Claim 38 (New). A prophylactic or therapeutic vaccine comprising an immunologically effective amount of a *H. pylori* CT polypeptide comprising SEQ ID NO:3, which polypeptide: (i) can induce the production of antibodies to *H. pylori* and (ii) exhibits substantially no toxicity, or substantially reduced toxicity.

Claim 39 (New). The vaccine of claim 38, further comprising an immunologically effective amount of a second polypeptide comprising *H. pylori* cytotoxin associated immunodominant (CAI) antigen or a fragment thereof, which second polypeptide: (i) comprises at least ten amino acids, (ii) can induce the production of antibodies to *H. pylori*, and (iii) exhibits no functional contribution to toxicity, or a substantially reduced functional contribution to toxicity.

Claim 40 (New). The vaccine of claim 39, wherein said second polypeptide comprises at least fifteen amino acids.

Claim 41 (New). A method of preparing a prophylactic or therapeutic vaccine comprising bringing into association:

(1) an immunologically effective amount of a *H. pylori* CT polypeptide, which polypeptide: (i) can induce the production of antibodies to *H. pylori* and (ii) exhibits substantially no toxicity, or substantially reduced toxicity, and

(2) a pharmaceutically acceptable carrier.

Claim 42 (New). The method of claim 41, further comprising adding an immunologically effective amount of a second polypeptide comprising *H. pylori* CAI antigen or fragment thereof, which second polypeptide: (i) comprises at least ten amino acids, (ii) can induce the production of antibodies to *H. pylori*, and (iii) exhibits no functional contribution to toxicity, or a substantially reduced functional contribution to toxicity.

Claim 43 (New). The method of claim 42, wherein the second polypeptide comprises SEQ ID NO:5, or a fragment thereof, which second polypeptide: (i) comprises at least ten amino acids, (ii) can induce the production of antibodies to *H. pylori*, and (iii) exhibits no functional contribution to toxicity, or a substantially reduced functional contribution to toxicity.

Claim 44 (New). A prophylactic or therapeutic vaccine comprising an immunologically effective amount of a recombinantly produced *H. pylori* CT polypeptide, wherein said recombinantly produced polypeptide (i) can induce the production of antibodies to *H. pylori* and (ii) exhibits substantially no toxicity, or substantially reduced toxicity, and a pharmaceutically acceptable carrier.

REMARKS

Claims 1 - 37 were pending.

By way of this amendment, claims 1 - 37 are canceled without prejudice, and new claims 38 - 44 are added.

Upon entry of this amendment claims 38 - 44 will be pending.

Summary of the Amendment

The specification has been amended to update the status of the priority and continuing applications. The specification has also been amended to identify the SEQ ID NOs appearing in the figures, and to provide proper SEQ ID NOs for the sequences appearing at page 49, line 39, page 50, line 1, and page 53, line 8. The specification has been amended to correct other informalities and typographical errors. Support for the amendment to the paragraph spanning from page 3, line 31 to page 4, line 6 can be found in original claims 8 and 9 of the application as filed. No new matter has been added.

An abstract has also been provided.

Claims 1 - 37 are canceled without prejudice.

New claims 38 - 44 are added to refer to specific embodiments of the invention. Support for new claims 38 - 44 is found in the original claims, and throughout the specification as filed. No new matter has been added.

New claims 38 - 44 recite prophylactic or therapeutic vaccines comprising CT polypeptides and methods of preparing them.

Conclusion

Applicants respectfully submit that claims 38 - 44 are in condition for allowance. A notice of allowance is earnestly solicited. The Examiner may call the undersigned at 215-557-5901 if a telephonic interview would be helpful.

Correspondence

All correspondence from the Patent and Trademark Office concerning this application should be sent to:

Alisa A. Harbin, Esq.
Vice President & Associate Chief Patent Counsel
Chiron Corporation
Intellectual Property - R440
P.O. Box 8097
Emeryville, CA 94608-8097

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached pages are captioned **"Version with markings to show changes made."**

Respectfully submitted,

Date: Aug. 2, 2001

Robin S. Quartin
Registration No. **45,028**

WOODCOCK WASHBURN KURTZ
MACKIEWICZ & NORRIS LLP
One Liberty Place - 46th Floor
Philadelphia, PA 19103
(215) 568-3100

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

Marked up versions of the paragraphs of the specification which are amended by replacement herein.

Paragraph spanning page 3, line 31 to page 4, line 6:

The present invention describes nucleotide and amino acid sequences for three major [H. pylori] *H. pylori* proteins. Specifically, these are the cytotoxin, the "Cytotoxin Associated Immunodominant" (CAI) antigen, and the heat shock protein. None of the complete amino acid sequences for these proteins has been known, nor have their genes been identified. The present invention pertains to not only these purified proteins and their genes, but also recombinant materials associated therewith, such as vectors and host cells. The present invention provides cytotoxin polypeptides that exhibit substantially no toxicity, or substantially reduced toxicity. The present invention also provides CAI and heat shock polypeptides that exhibit no functional contribution to toxicity, or a substantially reduced functional contribution to toxicity. The understanding at the molecular level of the nature and the role of these proteins and the availability of recombinant production has important implications for the development of new diagnostic for [H. pylori] *H. pylori* and for the design of vaccines that may prevent [H. pylori] *H. pylori* infection and treat disease.

Brief Description of the Drawings section at page 4:

[Fig. 1 is] Figs. 1A, 1B, and 1C (SEQ ID NO:2) comprise the nucleotide sequence for the cytotoxin (CT) protein.

Fig. 2 (SEQ ID NO:3) is the amino acid sequence for the cytotoxin (CT) protein.

Fig. 3 is a map of the [cai] *cai* gene for the CAI protein and summary of the clones used to identify and sequence this gene.

[Fig. 4 is] Figs. 4A through 4F (SEQ ID NO:4 and SEQ ID NO:5) comprise the nucleotide and amino acid sequences of the CAI antigen. The numbers along the left-hand margins of Figs. 4A, 4C, and 4E designate the amino acid positions, and the numbers along the right-hand margins of Figs. 4B, 4D, and 4F designate the nucleotide positions.

[Fig. 5 is] Figs. 5A, 5B, and 5C (SEQ ID NO:7 and SEQ ID NO:6) comprise the nucleotide and amino acid sequences of the heat shock protein (hsp).

Paragraph spanning page 49, line 30 to page 50, line 9:

DNA manipulation was performed using standard procedures. DNA sequencing was performed using Sequenase 2.0 (USB) and the DNA fragments shown in Fig. 3 subcloned in [Bluscript] Bluescript KS+. Each strand was sequenced at least three times. The region between nucleotides 1533 and 2289, for which a DNA clone was not available, was amplified by PCR and sequenced using asymmetric PCR, and direct sequencing of amplified products. The overlapping of this region, was confirmed by one and double side anchored PCR: an external universal anchor (5'-GCAAGCTTATCGATGTCGACTCGAGCT-3' (SEQ ID NO:1) / 5'-GACTCGAGTCGACATCGA-3' (SEQ ID NO:8)) containing a protruding 5' HindIII sequence, and the recognition sites of ClaI, SalI, XhoI, was ligated to primer-extended DNA and amplified. A second round of PCR using nested primers was then used to obtain fragments of DNA suitable for cloning and sequencing. DNA sequence data were assembled and analyzed with the GCG package (Genetics Computer Group, Inc., Madison, WI) running on a VAX 3900 under VMS. The GenBank and EMBL databases were examined using the EMBL VAXcluster.

Paragraph spanning page 52, line 15 to page 53, line 9:

The [cai] *cai* gene coded for a putative protein of 1147 amino acids, with predicted molecular weight of 128012.73 Daltons and an isoelectric point of 9.72. The basic properties of the

purified protein were confirmed by two dimensional gel electrophoresis. The codon usage and the GC content (37%) of the gene were similar to that described for other [H. pylori] *H. pylori* genes (13, 26). A putative ribosome binding site: AGGAG, was identified 5 base pairs upstream from the proposed ATG starting codon. Computer search for promoter sequences of the region upstream from the ATG start codon, identified sequences resembling either -10 or -35 regions, however, a region with good consensus to an [E. coli] *E. coli* promoter, or resembling published [H. pylori] *H. pylori* promoter sequences was not found. Primer extension analysis of purified [H. pylori] *H. pylori* RNA showed that 104 and 214 base pairs upstream from the ATG start codon there are two transcriptional starts sites. Canonical promoters could not be identified upstream from either transcriptional [E. coli] *E. coli* is also recognizing a promoter in this region, however, it is not clear whether [E. coli] *E. coli* recognizes the same promoters of [H. pylori] *H. pylori* or whether the [H. pylori] *H. pylori* DNA that is rich in A-T provides [E. coli] *E. coli* with regions that may act as promoters. A rho independent terminator was identified downstream from the stop codon. In Fig. 4, the AGGAG ribosome binding site and terminator are underlined, and the repeated sequence and motif containing 6 asparagines are boxed. The CAI antigen was very hydrophilic, and did not show obvious leader peptide or transmembrane sequences. The most hydrophilic region was from amino acids 600 to 900, where also a number of unusual features can be observed: the repetition of the sequences EFKNGKNKDFSK (SEQ ID NO:9) and EPYIA (SEQ ID NO:10), and the presence of a stretch of six contiguous asparagines (boxed in Fig. 4).

Paragraph at page 60, spanning lines 15 to 29:

The purified fusion protein was tested by Western blot using sera of patients infected by [H. pylori] *H. pylori* and affected by atrophic and superficial gastritis, and patients with duodenal and gastric ulcers: most of the sera recognized the recombinant protein. However, the degree of recognition greatly varied between different individuals and the antibody levels did not show any

obvious correlation with the type of disease. In addition, antibodies against [H. pylori] *H. pylori* antigens and in particular against hsp protein were found in most of the 12 sera of patients affected by gastric carcinoma that were tested. Although [H. pylori] *H. pylori* hsp recognition could not be put in relation with a particular clinical state of the disease given the high conservation between [H. pylori] *H. pylori* hsp and its human homolog, it is possible that this protein may induce autoimmune antibodies cross-reacting with the human counterpart. This class of homologous proteins has been implicated in the induction of autoimmune disorders in different systems. [Thenpresent] The presence of high titers of anti-[H. pylor] *H. pylori* hsp antibodies, potentially cross-reacting with the human homolog in dispeptic patients, suggests that this protein has a role in gastroduodenal disease. This autoreactivity could play a role in the tissue damage that occurs in [H. pylori] *H. pylori*-induced gastritis, thus increasing the pathogenic mechanisms involved in the infection of this bacterium.

In the claims:

Claims 38 - 44 have been added.